EJP
European Journal of Pain

ORIGINAL ARTICLE

Functional interaction between orexin-1 and CB1 receptors in the periaqueductal gray matter during antinociception induced by chemical stimulation of the lateral hypothalamus in rats

M.H. Esmaeili[1], Z. Reisi[2], S. Ezzatpanah[2], A. Haghparast[2]

1 Cellular and Molecular Research Center & Department of Physiology, Qazvin University of Medical Sciences, Qazvin, Iran
2 Neuroscience Research Center, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Correspondence
Abbas Haghparast
E-mails: Haghparast@yahoo.com;
Haghparast@sbmu.sc.ir

Funding sources
No funding sources are used in this study.

Conflicts of interest
None declared.

Accepted for publication
13 April 2016

doi:10.1002/ejp.899

Abstract

Background: Chemical stimulation of the lateral hypothalamus (LH) with carbachol induces antinociception which is antagonized by blockade of orexin receptors in some pain modulatory sites in the tail-flick test. In this study, we evaluated the role of orexin-1 and CB1 receptors in the periaqueductal gray matter (PAG), a critical pain modulatory site, in mediation of antinociceptive responses induced by LH stimulation in rats.

Methods: One hundred thirty-two adult male albino Wistar rats weighing 180–250 g were unilaterally implanted with two separate cannulae into the LH and ventrolateral PAG (vlPAG). Intra-vlPAG administration of SB334867, as a selective orexin-1 receptor antagonist (0.5, 1.5, 5, 15 and 50 nM), or AM251, as a selective CB1 receptor antagonist (1, 3, 10, 30 and 100 nM), was performed just 5 min before carbachol (125 nM) microinjection into the LH.

Results: Our findings showed that SB334867 or AM251 administration dose dependently prevented the development of LH-induced antinociception in rats. Treatment with two antagonists at the same time could not intensify their effects in comparison with separate administration of antagonists.

Conclusion: It seems that antinociceptive effect of intra-LH administration of carbachol is mediated, at least partially, through the activation of orexin-1 and CB1 receptors in the vlPAG.

Significance: This work demonstrates a pain modulatory role of the orexinergic system via the PAG in hypothalamic-mediated analgesia suggesting that orexins can be advantageously targeted to achieve analgesia.

What does this study add?: OX1 receptor antagonist (SB334867) administration into the ventrolateral periaqueductal gray matter (vlPAG) dose dependently blocked the carbachol-induced antinociception. CB1 receptor antagonist (AM251) microinjection in the vlPAG prevented carbachol-induced antinociception in a dose-dependent manner. Concurrent administration of SB334867 and AM251 into the vlPAG did not reinforce the antinociceptive responses.

1. Introduction

Orexin-A and orexin-B (also called hypocretin-1 and -2) are made exclusively in the perifornical area, lateral and posterior hypothalamus and send their projections throughout the central nervous system (CNS) (Lu et al., 2000; Marcus et al., 2001; Sadeghi et al., 2013). Orexins activate two groups of G-protein-coupled receptors (GPCRs): the orexin-1 and -2 receptors (Ox1r and Ox2r). Ox1r is selective for orexin-A, whereas Ox2r binds to both orexin-A and -B (Sakurai et al., 1998). The broad projections of orexinergic neurons carry implications for a variety of functions including control of feeding behaviour, sleep–wake cycle, cardiovascular function, hormone secretion and, more recently, the modulation of nociceptive processing (Ferguson and Samson, 2003; Samson et al., 2005; Azhdari-Zarmehri et al., 2011).

Several lines of evidence have shown that inactivation or electrical stimulation of the lateral hypothalamus (LH) produces antinociception (Carstens et al., 1983; Tasker et al., 1987). Also, intra-LH administration of carbachol, a cholinergic receptor agonist, glutamate or morphine leads to certain levels of antinociception in the acute (Behbehani et al., 1988; Holden and Naleway, 2001) and persistent inflammatory (Ezzatpanah et al., 2015) pain models. There is a consensus that LH-induced antinociception is directly or indirectly mediated through the periaqueductal gray matter (PAG) and rostroventromedial medulla (RVM), which ultimately triggers the activation of descending noradrenergic pathways (Behbehani et al., 1988). Like electrical stimulation, opioid administration into the ventrolateral part of PAG (vlPAG), which is considered to be important in pain modulation, produces antinociception (Lewis and Gebhart, 1977). Anatomical evidences have indicated that orexinergic projections and orexin receptors are distributed along all parts of pain circuitry, including the PAG (Peyron et al., 1998; Horvath et al., 1999; Cheng et al., 2003). Recently, it has been shown that orexin-A microinjection into the PAG reduces formalin-evoked nociceptive behaviours during the interphase and late phase but not during the early phase and intra-PAG administration of SB334867, a selective Ox1r antagonist, antagonized the antinociceptive effect of orexin-A (Azhdari-Zarmehri et al., 2011).

Also, previous studies have suggested that the PAG is an important site for endocannabinoid-mediated analgesia (Hohmann et al., 2005; Maione et al., 2006). Endocannabinoids, such as anandamide and 2-arachidonoylglycerol (2-AG), are lipid neuromodulators in the brain and activate cannabinoid receptors (CB1 and CB2), both of which modulate nociception (Samineni et al., 2011). The finding that intra-vlPAG microinjection of orexin-A can increase hot-plate latency in rats confirms that vlPAG is an important site of action for orexin-induced antinociception (Azhdari-Zarmehri et al., 2011; Ho et al., 2011). On the other hand, the antinociceptive effect of orexin-A was markedly reduced by cotreatment with AM251, a CB1 receptor antagonist (Ho et al., 2011). This finding suggests that there is an interaction between orexinergic and endocannabinoid systems in the pain modulatory role of PAG. Accordingly, in this study we aimed to examine the involvement of PAG orexin-1 and CB1 receptors in mediation of antinociception induced by intra-LH carbachol administration in the tail-flick test as an animal model of acute pain.

2. Materials & Methods

2.1 Subjects

One hundred thirty-two adult male albino Wistar rats (Pasteur Institute, Tehran, Iran) weighing 180–250 g were used in these experiments. The rats were kept in a vivarium maintained at a 12/12 h light/dark cycle at room-controlled temperature (23 ± 1 °C) with free access to food and water. Rats were habituated to their new environment and handled for a week before the beginning of experimental procedure. The animals were randomly allocated to different experimental groups. Each animal was used only once. All experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996) and were approved by the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences.

2.2 Stereotaxic surgery

Rats were anesthetized with intraperitoneal injection of ketamine 10% (100 mg/kg) and xylazine 2% (10 mg/kg). Animals were placed in the stereotaxic apparatus (Stoelting Company, Wood Dale, IL, USA). The coordinates for the LH and vlPAG regions were determined by the rat brain atlas (Paxinos and Watson, 2007); vlPAG: AP = 7.75 ± 0.15 mm caudal to bregma; Lat = ±0.5 mm; DV = 5.7 mm ventral from the skull surface (23-gauge cannula, 9 mm length) and LH: AP = 2.65 ± 0.15 mm caudal to bregma, Lat = ±1.6 mm and DV = 8.8 mm ventral from the

skull surface for the LH (23-gauge cannula, 12 mm length). Two small holes were drilled in the skull and stainless cannulae were unilaterally implanted into the LH and vlPAG (left LH and vlPAG or right LH and vlPAG), 1 mm above the appropriate injection place. The guide cannulae were secured in their places using two stainless steel screws and dental acryl cement. After the cement was completely dried and hardened, two stainless steel stylets were used to seal the guide cannulae during recovery period. A single dose of penicillin-G 200,000 IU/mL (0.2–0.3 mL/rat, intramuscular) was administered immediately after surgery. Animals were individually housed and allowed to recover for a period of 5–7 days before the experiments.

2.3 Drugs and drug administration

Carbachol (Sigma-Aldrich, St. Louis, MO, USA) was used as a cholinergic agonist and dissolved in the normal saline to prepare the 25 and 125 nM solutions. SB334867 (Tocris Bioscience, Bristol, UK), as an Ox1r antagonist, and AM251 [1-(2,4-dichlorophenyl)5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide], as a CB1 receptor antagonist (Sigma-Aldrich, Steinheim, Germany), were dissolved in the dimethyl sulfoxide (DMSO; Sigma-Aldrich) and appropriate solutions of SB334867 (1, 3, 10, 30 and 100 nM) or AM251(0.5, 1.5, 5, 15 and 50 nM) were prepared. All drugs were freshly prepared on the experiment day.

Microinjections were performed by lowering a stainless steel injector (a 30-gauge needle which was 1 mm longer than the guide cannulae) into the vlPAG and/or LH. The injector was attached to a 1-μL Hamilton syringe by a polyethylene microtube (PE-20). Drug microinjection (DMSO or antagonist) into the vlPAG (in a total volume of 0.2 μL) was performed 5 min before intra-LH administration of 0.5 μL of saline or carbachol. The drug solutions or vehicles were infused unilaterally over 60 s and was left for an extra 60 s to facilitate the drug or vehicle diffusion. During the infusion procedure, rats could freely move and all efforts were made to reduce animal stress.

2.4 Tail-flick test

Tail-flick test is used as a model for studying the acute pain. The nociceptive threshold was measured by the tail-flick apparatus (Harvard Apparatus, Cambridge, UK). The reaction time between the onset of heat stimulus and the movement of tail was determined by an automatic sensor as tail-flick latency (TFL). The light source was set at an intensity that yields baseline TFL values in the range 3–4 s (about 45% of maximal light intensity). If the animal failed to flick its tail within 10 s (cut-off point), the tail was removed from the coil to prevent damage to the skin (Ebrahimzadeh and Haghparast, 2011; Parvishan et al., 2011; Haghparast et al., 2012). In each experiment, after measurement of baseline TFL values and administration of appropriate treatment, the heat was applied in succession 3, 5 and 7 cm from the caudal tip of tail. The average of three consecutive post-drug TFLs, with time intervals of 30–40 s, was used for measurement of percentage of maximal possible effect (%MPE). %MPE was measured for each 5, 15, 30, 45 and 60 time points. TFLs (sec) are expressed either as raw post-drug TFL or %MPE. In this study, we used %MPE which was calculated from the following formula:

$$\%\mathrm{MPE} = \frac{\text{Post-drug TFL (sec)} - \text{Baseline TFL (sec)}}{\text{Cut-off value (sec)} - \text{Baseline TFL (sec)}} \times 100$$

2.5 Experimental design

2.5.1 Dose–response effects of carbachol microinjection into the LH in tail-flick test

In this set of experiments, carbachol solutions (25 and 125 nM) were microinjected into the LH before the tail-flick test, whereas control animals received saline into the LH instead of carbachol.

2.5.2 Effects of intra-vlPAG administration of OX1 receptor antagonist on the antinociception induced by carbachol injection into the LH

To test the role of OX1 receptors located within the vlPAG during the LH stimulation-induced antinociception in tail-flick test, baseline TFLs were measured. Then, 0.5, 1.5, 5, 15 and 50 nM solutions of SB334867 were unilaterally injected into the vlPAG 5 min prior to intra-LH administration of carbachol (125 nM). The procedure was followed by the tail-flick test after 5 min. In the vehicle group, DMSO was microinjected into the vlPAG and 2–4 min later animals received saline into the LH. The DMSO group received DMSO and carbachol into the vlPAG and LH before the test, respectively.

2.5.3 Effects of intra-vlPAG administration of CB1 receptor antagonist on the antinociception induced by carbachol injection into the LH

To examine the role of intra-vlPAG CB1 receptors in the LH stimulation-induced antinociception, 1, 3, 10, 30 and 100 nM solutions of AM251 were

unilaterally injected into the vlPAG 5 min prior to the infusion of carbachol (125 nM), which was followed by the tail-flick test after 5 min. In the vehicle group, DMSO was microinjected into the vlPAG and 2–4 min later, animals received saline into the LH. The DMSO group was microinjected with DMSO and carbachol into the vlPAG and LH before the test, respectively.

2.5.4 Interaction of intra-vlPAG administration of OX1 receptor antagonist and CB1 receptor antagonist on the antinociception induced by administration of carbachol into the LH

In the last set of experiments, to examine the interaction of intra-vlPAG OX1 receptors and CB1 receptors in the LH stimulation-induced antinociception, 0.5 nM SB334867 and 1 nM AM251 or 5 nM SB334867 and 10 nM AM251 were injected into the vlPAG, 5 min prior to the infusion of carbachol (125 nM). The procedure was followed by tail-flick test after 5 min. In the vehicle group, 2–4 min before intra-LH administration of saline, DMSO was microinjected into the vlPAG. The DMSO and carbachol were microinjected into the vlPAG and LH of the DMSO group before the test.

The effect of carbachol injection into some brain regions surrounding the LH or the effect of carbachol administration into the LH in combination with 50 nM solution of SB334867 or 100 nM solution of AM251 into some brain regions surrounding the vlPAG was also examined to distinguish between the results specific to drug injections into the LH and vlPAG and those obtained from drug injections into the neighbouring regions.

2.6 Histology

The animals were deeply anesthetized with ketamine and xylazine after the experiments were accomplished. Transcardial perfusion was carried out with 0.9% saline and 10% formalin solution. The brains were removed and cut coronally in 50 μm sections through the cannulae placements. The neuroanatomical locations of cannulae tips were confirmed using Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007). Only the animals with correct cannulae placements were included in the results (Fig. 1).

2.7 Statistics

The results attained are expressed as mean ± SEM (standard error of mean). The mean maximal possible effect (%MPE) of drugs as an analgesic index was subjected to two-way ANOVA followed by Bonferroni's test for multiple comparisons. To evaluate the nociceptive responses, AUC was calculated as raw pain scores × time by linear trapezoidal method and a single value was used in statistical analyses. The calculated AUC and pain score values in all groups were subjected to one-way repeated measures ANOVA followed by protected Newman–Keuls test for multiple comparisons. Unpaired student *t*-test was used for comparison of AUC values of two independent groups. The differences with *p*-values less than 0.05 were considered to be statistically significant.

3. Results

In each set of experiments, one-way ANOVA showed that there are not any significant differences among the baseline TFLs of animals. However, intra-vlPAG administration of different solutions of SB334867, a selective OX1 receptor antagonist, or AM251, a CB1 receptor antagonist, changed the TFLs. The effect of SB334867 or AM251 administration on LH-induced antinociception was compared to the vehicle and DMSO groups as controls.

3.1 Dose–response effects of chemical stimulation of the LH by carbachol on the tail-flick test

In this experiment, we examined a dose–response effect of carbachol solutions (25 and 125 nM) microinjected into the LH on TFLs in the tail-flick test in rats. Two-way ANOVA followed by Bonferroni's test [treatment effect: $F(2,65) = 69.39$, $p < 0.0001$; time effect: $F(4,65) = 0.5898$, $p = 0.6712$; interaction effect: $F(8,65) = 0.4114$, $p = 0.9101$; Fig. 2A] affirmed that there are significant differences in %MPEs as an index of antinociception between the control and experimental group (125 nM). In other words, carbachol at the dose of 125 nM caused antinociception which is seen as a significant increase in %MPEs.

Furthermore, one-way ANOVA followed by Dunnett's multiple comparison test [$F(2,15) = 29.04$, $p < 0.0001$] showed significant increase in %MPEs in carbachol group (125 nM) compared to the group which had received 25 nM solution of carbachol and control group ($p < 0.001$) (Fig. 2B). Therefore, 125 nM solution of carbachol was used in other set of experiments.

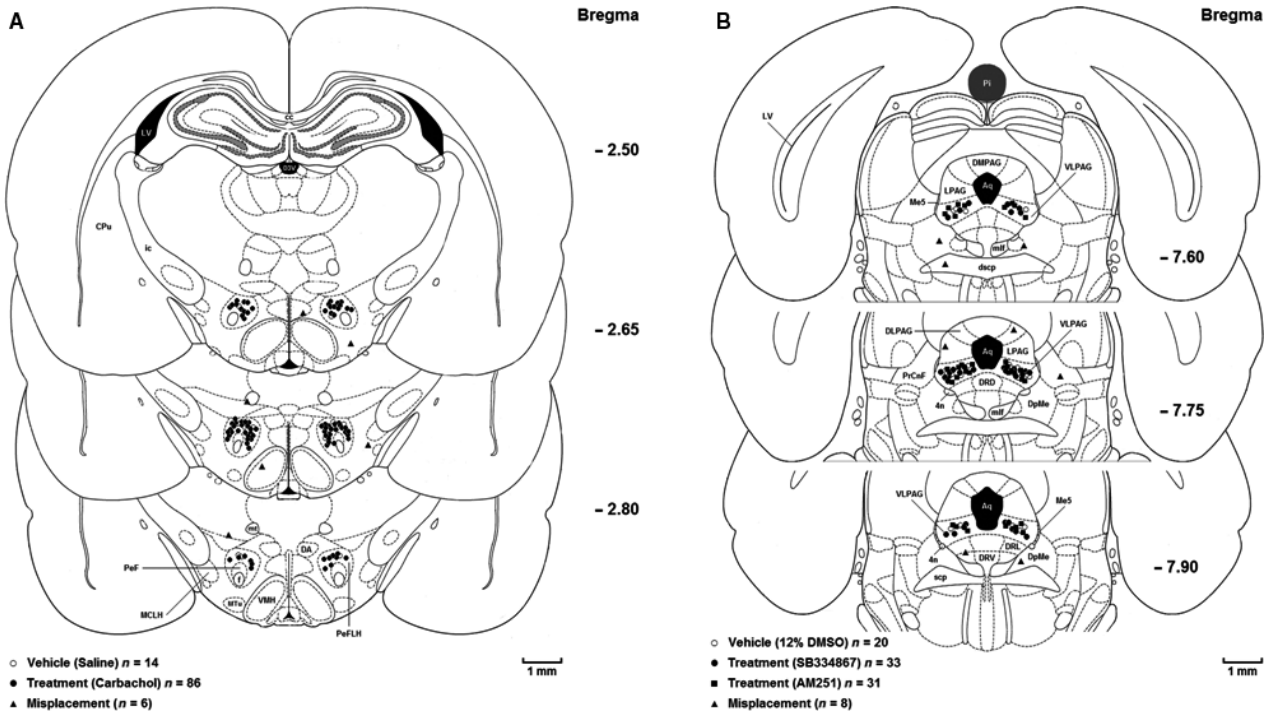

Figure 1 Three coronal schematic microinjection sites (A) in the LH (○ = saline microinjection and ● = carbachol microinjection and ▲ = misplacement) and (B) in the PAG (○ = DMSO microinjection and ● = SB334867 and ■ = AM251 microinjection and ▲ = misplacement). All microinjections were performed unilaterally. LV, lateral ventricle; D3V, dorsal third ventricle; cc, corpus callosum; ic, internal capsule; CPu, caudate putamen striatum; mt, mammillothalamic tract; DA, dorsal hypothalamic area; PeF, perifornicalnucleus; f, fornix; MCLH, magnocellular nucleus of lateral hypothalamus; MTu, medial tuberal nucleus; VMH, ventromedial hypothalamic nucleus; PeFLH perifornical part of lateral hypothalamus; Pi, pineal gland; DMPAG, dorsomedial periaqueductal area; LPAG, lateral periaqueductal gray; VLPAG, ventrolateral periaqueductal gray; Aq, aqueduct; Me5, mesencephalic trigeminal nucleus; mlf, medial langitudinalfasciculus; dscp, decussation of the superior cerebellar; DLPAG, dorsolateral periaqueductal gray; DRD, dorsal raphe nucleus (dorsal part); PrCnF, precuneiform area; 4n, trochlear nerve; DpMe, deep mesencephalic nucleus; DRL, dorsal raphe nucleus (later part); DRV, dorsal raphe nucleus (ventral part); scp, superior cerebellar peduncle. Scale bar is 1 mm. In all rats cannulae were placed in the left or right side.

3.2 Effect of intra-vlPAG administration of OX1 receptor antagonist on the LH stimulation-induced antinociception

Two-way ANOVA followed by Bonferroni's test indicated significant differences in antinociceptive responses in DMSO group and groups which had received carbachol following different solutions of SB334867compared to the vehicle group [treatment effect: F (6,175) = 59.04, $p < 0.0001$; time effect: F (4,175) = 4667, $p = 0.0013$; interaction effect: F (24,175) = 0.5271, $p = 0.9666$; Fig. 3A].

As it has been shown in Fig. 3B, one-way ANOVA followed by Newman–Keuls multiple comparison test indicated that there are significant differences in AUC values calculated for %MPEs [F (6,41) = 29.62, $p < 0.0001$] among the experimental and control (vehicle and DMSO) groups. Data gained from these experiments showed that intra-vlPAG administration of SB334867 dose dependently prevents LH stimulation-induced antinociception in rats. Intra-vlPAG administration of 5, 15 and 50 nM solutions of SB334867 (but not 0.5 and 1.5 nM) significantly reduced AUC values in comparison with DMSO control group which had received carbachol into the LH ($p < 0.01$ and $p < 0.001$, respectively; Fig. 3B).

3.3 Effect of intra-vlPAG administration of CB1 receptor antagonist on the LH stimulation-induced antinociception

A dose–response effect of intra-vlPAG administration of selective CB1 receptor antagonist, AM251, on the antinociceptive response of intra-LH administration of carbachol during a 60-min period was observed in the following experiments. Two-way ANOVA followed by Bonferroni's test [Treatment effect: F (6,175) = 49.57, $p < 0.0001$; Time effect: F (4,175)

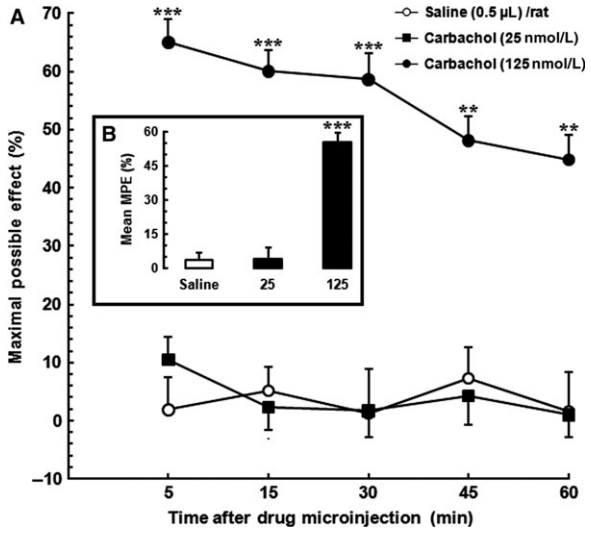

Figure 2 Effect of unilateral administration of 25 and 125 nM solutions of carbachol in the lateral hypothalamus (LH) on tail-flick latencies in rats. (A) Maximal possible effect of two doses of carbachol 5, 15, 30, 45 and 60 min after microinjection and (B) area under the curves (AUCs) calculated for %MPEs in 60-min period in tail-flick test as a model of acute pain shown in A. Administration of 25 and 125 nM solutions of carbachol reduced nociceptive responses compared to the control group. Each point shows the mean ± SEM for 5–6 rats. $^{**}p < 0.01$, $^{***}p < 0.001$ different from the saline control group.

= 5.35, $p = 0.0004$; Interaction: F (24,175) = 0.2766, $p = 0.979$] revealed that there are significant differences in %MPE values among the experimental and control (vehicle and DMSO) groups (Fig. 4A).

In addition, one-way ANOVA followed by Newman–Keuls multiple comparison test [F (6,41) = 29.31: $p < 0.0001$] indicated significant differences in AUC values calculated for %MPEs among the experimental and control groups. The results showed that intra-vlPAG administration of AM251 prevents LH stimulation-induced antinociception in rats in a dose-dependent manner. In this respect, intra-vlPAG administration of 10, 30 and 100 nM solutions of AM251 (but not 1 and 3 nM) could significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) reduce AUC values in comparison to DMSO control group (Fig. 4B).

3.4 The effect of concurrent administration of SB334867 and AM251 into the vlPAG on the antinociception induced by carbachol microinjection into the LH

To examine the interaction between the orexinergic and cannabinoid systems in the vlPAG on the antinociceptive response of carbachol, animals

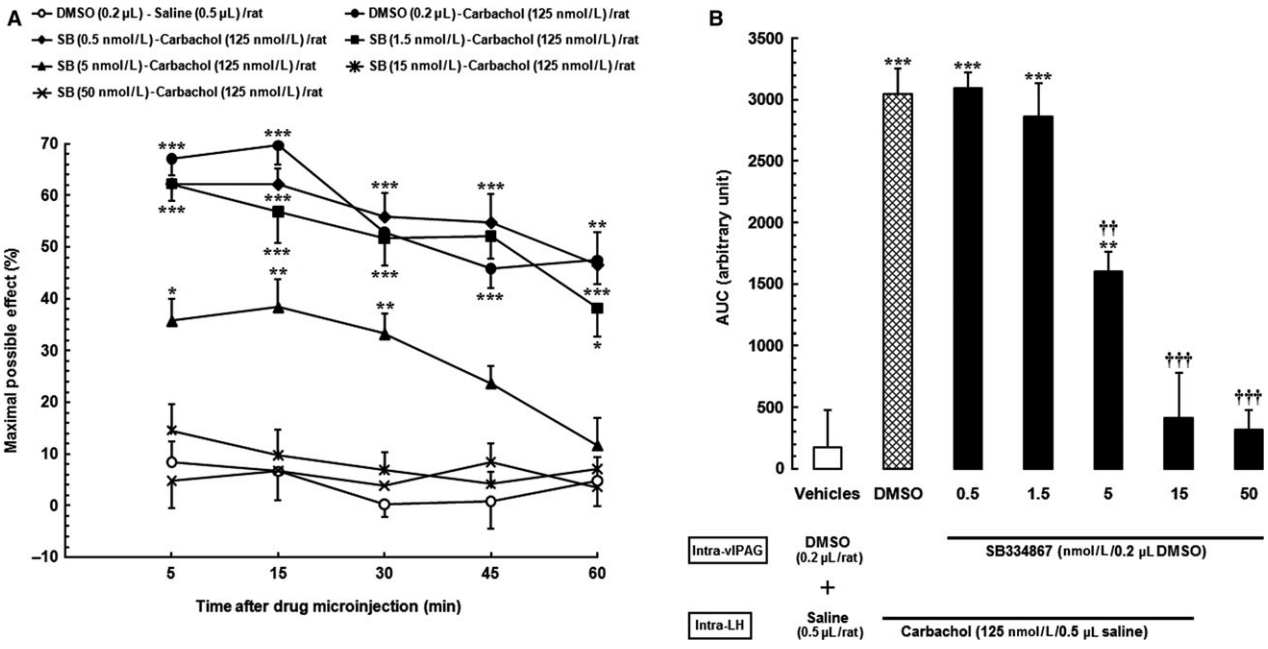

Figure 3 Effect of unilateral microinjection of different solutions of SB334867, an OX1 receptor antagonist, in the ventrolateral periaqueductal gray matter (vlPAG) on the antinociception induced by chemical stimulation of the lateral hypothalamus (LH). (A) maximal possible effect 5, 15, 30, 45 and 60 min after drug microinjection and (B) area under the curves (AUCs) calculated for %MPEs in 60-min period in tail-flick test as a model of acute pain shown in A. Administration of 0.5, 1.5, 5, 15 and 50 nM solutions of SB3348675 min before intra-LH microinjection of carbachol (125 nM) reduced LH-induced analgesia in a dose-dependent manner. Each point shows the mean ± SEM for 5–7 rats. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ different from the control (vehicle) group. $^{††}p < 0.01$, $^{†††}p < 0.01$ different from the DMSO group.

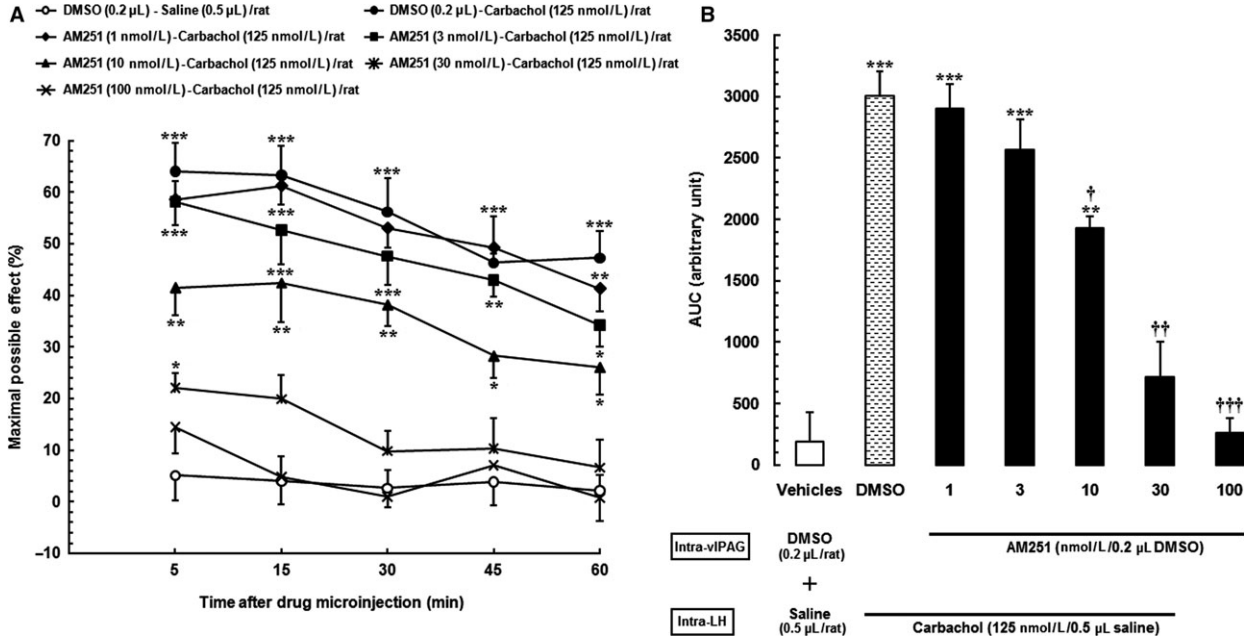

Figure 4 Effect of unilateral microinjection of different solutions of AM251, a CB1 receptor antagonist, in the ventrolateral periaqueductal gray matter (vlPAG) on the antinociception induced by chemical stimulation of the lateral hypothalamus (LH). (A) maximal possible effect 5, 15, 30, 45 and 60 min after microinjection and (B) area under the curves (AUCs) calculated for %MPEs in 60-min period in tail-flick test as a model of acute pain are shown in A. Administration of 1, 3, 10, 30 and 100 nM solutions of AM2515 min before intra-LH microinjection of carbachol (125 nM) reduced LH-induced analgesia in a dose-dependent manner. Each point shows the mean ± SEM for 5–8 rats. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ different from the control (vehicle) group. †$p < 0.05$, ††$p < 0.01$, †††$p < 0.01$ different from the DMSO group.

received two different doses of SB334867 and AM251 into the vlPAG, 5 min prior to carbachol infusion into the LH. Two-way repeated measures ANOVA followed by Bonferroni's test for %MPEs indicated significant differences in antinociceptive responses in DMSO group and groups which had concurrently received carbachol and different doses of SB334867 and AM251 compared to the control vehicle group [Treatment effect: F (3,140) = 76.61, $p < 0.0001$; Time effect: F (4,140) = 5.056, $p = 0.008$; Interaction effect: F (12,140) = 0.802, $p = 0.6479$; Fig. 5A]. Similarly, as shown in Fig. 5B, one-way ANOVA followed by Newman–Keuls multiple comparison test showed significant differences in AUC values calculated for %MPEs [F (3,31) = 38.22: $p < 0.0001$] among the experimental and control (vehicle and DMSO) groups. Data obtained from these experiments revealed that intra-vlPAG administration of SB334867 and AM251 at the doses of 5 and 10 nM significantly reduced the %MPEs and AUC values ($p < 0.01$) and reduced the LH stimulation-induced antinociception in rats compared to the DMSO group, but concurrent intra-vlPAG administration of lower doses of SB334867 (0.5 nM) and AM251 (1 nM) did not reduce the %MPEs and AUC values in comparison to the DMSO group (Fig. 5B). Furthermore, unpaired student t-test showed that AUC value in group which in addition to carbachol had received higher doses of antagonists was significantly more than that which had received carbachol and lower doses of SB334867 and AM251 ($p < 0.01$).

The results obtained from carbachol injection into some areas close to the LH were significantly different from those of DMSO group. In other words, it did not have any antinociceptive effect. Also, administration of 50 nM solution of SB334867 or 100 nM solution of AM251 into the some areas close to the vlPAG before intra-LH administration of carbachol could not antagonize LH-induced antinociception. Accordingly, it can be assumed that observed results are most likely due to effect of drug administration into the LH and vlPAG not neighbouring areas.

4. Discussion

The purpose of this study was to evaluate the involvement of orexin-1 and CB1 receptors in the vlPAG in antinociceptive responses induced by LH stimulation in rat. The major findings were as follows: (1) administration of 25 and 125 nM solutions of carbachol into the LH produced antinociceptive

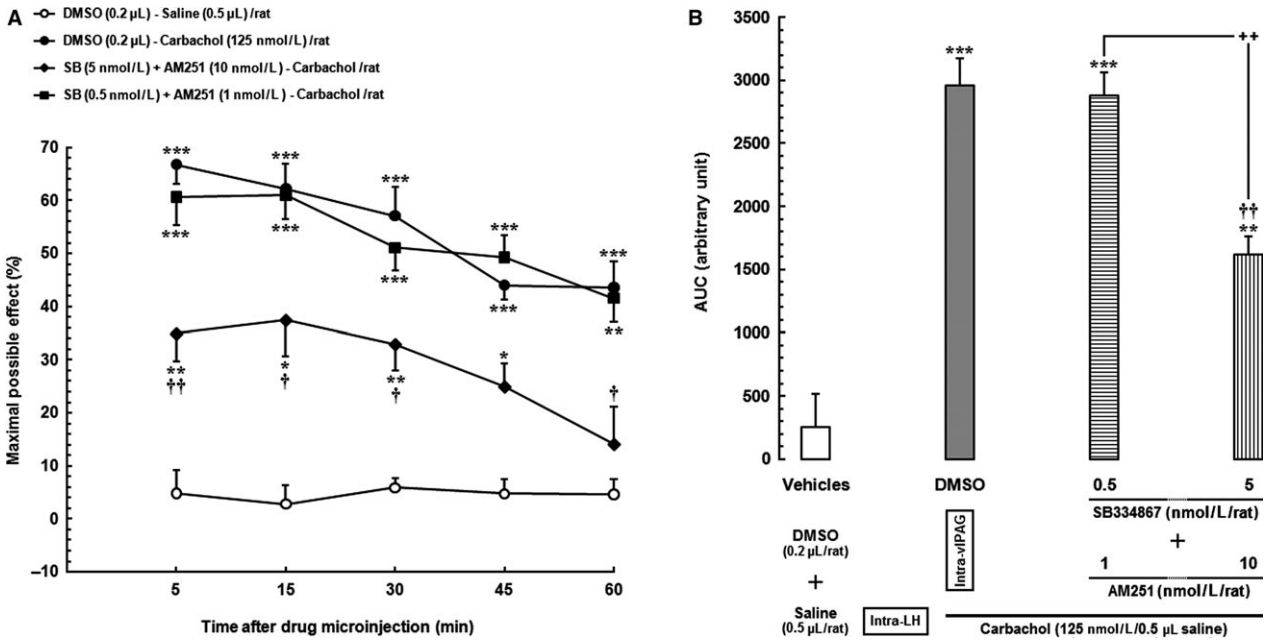

Figure 5 Effect of unilateral microinjection of two doses of SB334867, an OX1 receptor antagonist, and two doses of AM251, a CB1 receptor antagonist, in the ventrolateral periaqueductal gray matter (vlPAG) on the antinociception induced by chemical stimulation of the lateral hypothalamus (LH). (A) maximal possible effect 5, 15, 30, 45 and 60 min after microinjection and (B) area under the curves (AUCs) calculated for %MPEs in 60-min period in tail-flick test as a model of acute pain shown in A. SB334867 (0.5 and 5 nM) and AM251 (1 and 10 nM) administration 5 min before intra-LH microinjection of carbachol (125 nM) reduced LH-induced analgesia. Each point shows the mean ± SEM for eight rats. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ different from the control (vehicle) group. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ different from the DMSO group. $++p < 0.01$ different from two treatment groups.

responses in the tail-flick test, (2) an OX1 receptor antagonist (SB334867) administration into the vlPAG dose dependently blocked the carbachol-induced antinociception, (3) a CB1 receptor antagonist (AM251) microinjection in the vlPAG prevented carbachol- induced antinociception in a dose-dependent manner and (4) concurrent administration of SB334867 and AM251 into the vlPAG did not reinforce the antinociceptive responses.

Several studies have shown that the stimulation or inactivation of LH produces antinociception (Behbehani et al., 1988; Safari et al., 2009). The results of first set of experiments in this study agreed with the results of previous study showing that the chemical stimulation of LH induces analgesia (Sadeghi et al., 2013). In this study, two different solutions of carbachol were used: 25 and 125 nM. Carbachol solution of 125 nM significantly caused antinociceptive responses; hence, it was considered as the effective dose in following experiments.

Neuroanatomical studies have suggested that there is a physical association between LH and PAG. In this respect, Behbehani et al. (1988) have indicated that the majority of PAG neurons were excited and tail-flick latency increased in response to the electrical stimulation of LH. Furthermore, Peyron et al. (1998) suggested that a relative high density of orexinergic fibres project from the LH to the PAG and finally terminate within the RVM and dorsal horn of spinal cord (Peyron et al., 1998). There is an increasing amount of evidence indicating that orexinergic system is involved in pain modulation. The finding that intra-PAG microinjection of orexin-A increased hot-plate latency in rat (Azhdari-Zarmehri et al., 2011) confirms that PAG is an important site of action for orexin-induced antinociception. In addition, previous studies have reported that the systemic administration of orexin-A produced a dose-related analgesia in hot-plate test in rats and the intrathecal delivery of orexin-A-inhibited pain perception (Bingham et al., 2001). In view of the fact that the pain modulatory role of LH is mediated directly or indirectly through the PAG (Millan, 2002), it can be suggested that the orexinergic pathway from the LH to the PAG may be implicated in control of pain (Azhdari-Zarmehri et al., 2011).

Given that carbachol increases the firing of orexinergic system (Yamanaka et al., 2003), we attempted to assess the role of intra-vlPAG orexin receptors on the antinociception induced by carbachol stimulation

of LH. This study showed that carbachol causes antinociceptive behaviours. The antinociceptive effect of LH may be due to activation of orexin receptors distributed in the different brain regions involved in pain modulation, but reduction in analgesia after intra-vlPAG administration of orexin-1 receptor antagonist indicated that LH analgesia was at least partially mediated through the PAG orexin-1 receptors. It seems that orexins may directly excite and depolarize the PAG neurons; an effect that results in either the direct activation of RVM and dorsal horn of the spinal cord or activation of ascending pain modulatory pathways (Morgan et al., 1989). Endocannabinoid mechanisms are strongly implicated in endogenous antinociceptive mechanisms (Hohmann et al., 2005; Suplita et al., 2005). The release of endocannabinoids has been previously observed following the induction of seizures by convulsant drugs (Wallace et al., 2003). Earlier studies have suggested that intra-PAG CB1 receptors modulate local inhibitory networks to regulate nociception (Wallace et al., 2003). It seems that CB1 receptor-induced analgesia is probably mediated through the disinhibition of PAG GABAergic interneurons which exert an intrinsic inhibitory tone on PAG neurons (Pan et al., 1990; Meng et al., 1998; Vaughan et al., 1999; Samineni et al., 2011). In this study, intra-PAG administration of AM251, a CB1 antagonist, dose dependently prevented the LH-induced antinociception in rats. Given the fact that activation of GPCRs like OX1r results in synthesis of endocannabinoids (Ho et al., 2011), LH-induced analgesia may be due to disinhibition of GABAergic interneurons and indirect excitation of PAG neurons. In support of this finding, Ho et al. (2011) have shown that the antinociceptive effect of orexin-A was markedly reduced by cotreatment with AM251. This supports the idea that CB1 receptors are probably implicated in orexin-induced analgesia whether they are administrated directly into the PAG or released into the PAG after LH stimulation. So, it can be concluded that orexin-1 and CB1 receptors located in the PAG play a major role in LH-induced antinociception in the tail-flick test. In other words, the pain modulatory role of LH is partially mediated through the interaction between the orexinergic and cannabinoid systems in the PAG.

According to previous findings, SB334867 alone had no effect on TFLs compared to control group and there was no tonic orexin-1 receptor-mediated inhibitory system in the ventral tegmental area and nucleus accumbens in the tail-flick test (Sadeghi et al., 2013). On the other hand, it is assumed that cholinergic input from the basal forebrain neurons to the orexinergic neurons might be implicated not only in regulation of awake status but also in control of stressful situations (Tsujino and Sakurai, 2013). In this respect, previous study has demonstrated that prepro-orexin (precursor of orexin-A and -B) knockout mice showed less stress-induced analgesia; demonstrating that orexin release in stressful situation is involved in reduction in nociception (Watanabe et al., 2005). Thus, although there may not be a tonic orexin-1 receptor-mediated inhibitory system in the PAG in the tail-flick test, a hypothesis that needs more investigation, LH releases orexin when encountered with emotional and stressful situations.

Author contributions

Substantial contributions to conception and design: Abbas Haghparast; Acquisition, Analysis and interpretation of data: Mohammad Hossein Esmaeili and Abbas Haghparast; Drafting the article: Zahra Reisi and Somayeh Ezzatpanah; Revising the article critically for important intellectual content: Abbas Haghparast and Mohammad Hossein Esmaeili; Final approval of the version to be published: Abbas Haghparast.

Acknowledgement

The authors would like to thank the Department of Physiology, School of Medical Sciences, Qazvin University of Medical Sciences, Qazvin, Iran.

References

Azhdari-Zarmehri, H.A., Semnanian, S., Fathollahi, Y., Erami, E., Khakpay, R., Azizi, H., Rohampour, K. (2011). Intra-periaqueductal gray matter microinjection of orexin-A decreases formalin-induced nociceptive behaviors in adult male rats. *J Pain* 12, 280–287.

Behbehani, M.M., Park, M.R., Clement, M.E. (1988). Interactions between the lateral hypothalamus and the periaqueductal gray. *J Neurosci* 8, 2780–2787.

Bingham, S., Davey, P.T., Babbs, A.J., Irving, E.A., Sammons, M.J., Wyles, M., Jeffrey, P., Cutler, L., Riba, I., Johns, A., Porter, R.A., Upton, N., Hunter, A.J., Parsons, A.A. (2001). Orexin-A, an hypothalamic peptide with analgesic properties. *Pain* 92, 81–90.

Carstens, E., Fraunhoffer, M., Suberg, S. (1983). Inhibition of spinal dorsal horn neuronal responses to noxious skin heating by lateral hypothalamic stimulation in the cat. *J Neurophysiol* 50, 192–204.

Cheng, J.K., Chou, R.C.C., Hwang, L.L., Chiou, L.C. (2003). Antiallodynic effects of intrathecal orexins in a rat model of postoperative pain. *J Pharmacol Exp Ther* 307, 1065–1071.

Ebrahimzadeh, M., Haghparast, A. (2011). Analgesic effects of cannabinoid receptor agonist WIN55, 212-2 in the nucleus cuneiformis in animal models of acute and inflammatory pain in rats. *Brain Res* 1420, 19–28.

Ezzatpanah, S., Babapour, V., Sadeghi, B., Haghparast, A. (2015). Chemical stimulation of the lateral hypothalamus by carbachol attenuated the formalin-induced pain behaviors in rats. *Pharmacol Biochem Behav* 129, 105–110.

Ferguson, A.V., Samson, W.K. (2003). The orexin/hypocretin system: A critical regulator of neuroendocrine and autonomic function. *Front Neuroendocrinol* 24, 141–150.

Haghparast, A., Ghalandari-Shamami, M., Hassanpour-Ezatti, M. (2012). Blockade of D1/D2 dopamine receptors within the nucleus accumbens attenuated the antinociceptive effect of cannabinoid receptor agonist in the basolateral amygdala. *Brain Res* 1471, 23–32.

Ho, Y.C., Lee, H.J., Tung, L.W., Liao, Y.Y., Fu, S.Y., Teng, S.F., Liao, H.T., Mackie, K., Chiou, L.C. (2011). Activation of orexin 1 receptors in the periaqueductal gray of male rats leads to antinociception via retrograde endocannabinoid (2-arachidonoylglycerol)-induced disinhibition. *J Neurosci* 31, 14600–14610.

Hohmann, A.G., Suplita, R.L., Bolton, N.M., Neely, M.H., Fegley, D., Mangieri, R., Krey, J.F., Walker, J.M., Holmes, P.V., Crystal, J.D. (2005). An endocannabinoid mechanism for stress-induced analgesia. *Nature* 435, 1108–1112.

Holden, J.E., Naleway, E. (2001). Microinjection of carbachol in the lateral hypothalamus produces opposing actions on nociception mediated by α 1- and α 2-adrenoceptors. *Brain Res* 911, 27–36.

Horvath, T.L., Peyron, C., Diano, S., Ivanov, A., Aston-Jones, G., Kilduff, T.S., van den Pol, A.N. (1999). Hypocretin (orexin) activation and synaptic innervation of the locus coeruleus noradrenergic system. *J Comp Neurol* 415, 145–159.

Lewis, V.A., Gebhart, G. (1977). Evaluation of the periaqueductal central gray (PAG) as a morphine-specific locus of action and examination of morphine-induced and stimulation-produced analgesia at coincident PAG loci. *Brain Res* 124, 283–303.

Lu, X.Y., Bagnol, D., Burke, S., Akil, H., Watson, S.J. (2000). Differential distribution and regulation of OX1 and OX2 orexin/hypocretin receptor messenger RNA in the brain upon fasting. *Horm Behav* 37, 335–344.

Maione, S., Bisogno, T., de Novellis, V., Palazzo, E., Cristino, L., Valenti, M., Petrosino, S., Guglielmotti, V., Rossi, F., Di Marzo, V. (2006). Elevation of endocannabinoid levels in the ventrolateral periaqueductal grey through inhibition of fatty acid amide hydrolase affects descending nociceptive pathways via both cannabinoid receptor type 1 and transient receptor potential vanilloid type-1 receptors. *J Pharmacol Exp Ther* 316, 969–982.

Marcus, J.N., Aschkenasi, C.J., Lee, C.E., Chemelli, R.M., Saper, C.B., Yanagisawa, M., Elmquist, J.K. (2001). Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol* 435, 6–25.

Meng, I.D., Manning, B.H., Martin, W.J., Fields, H.L. (1998). An analgesia circuit activated by cannabinoids. *Nature* 395, 381–383.

Millan, M.J. (2002). Descending control of pain. *Prog Neurobiol* 66, 355–474.

Morgan, M.M., Sohn, J.H., Liebeskind, J.C. (1989). Stimulation of the periaqueductal gray matter inhibits nociception at the supraspinal as well as spinal level. *Brain Res* 502, 61–66.

Pan, Z., Williams, J., Osborne, P. (1990). Opioid actions on single nucleus raphe magnus neurons from rat and guinea-pig in vitro. *J Physiol* 427, 519–532.

Parvishan, A., Taslimil, Z., Ebrahimzadeh, M., Haghparast, A. (2011). Capsazepine, a transient receptor potential vanilloid type 1 (TRPV1) antagonist, attenuates antinociceptive effect of CB1 receptor agonist, WIN55, 212-2, in the rat nucleus cuneiformis. *Basic Clin Neurosci* 2, 19–26.

Paxinos, G., Watson, C. (2007). *The Rat Brain in Stereotaxic Coordinates* (San Diego: Elsevier Academic Press).

Peyron, C., Tighe, D.K., Van Den Pol, A.N., De Lecea, L., Heller, H.C., Sutcliffe, J.G., Kilduff, T.S. (1998). Neurons containing hypocretin (orexin) project to multiple neuronal systems. *J Neurosci* 18, 9996–10015.

Sadeghi, S., Reisi, Z., Azhdari-Zarmehri, H., Haghparast, A. (2013). Involvement of orexin-1 receptors in the ventral tegmental area and the nucleus accumbens in antinociception induced by lateral hypothalamus stimulation in rats. *Pharmacol Biochem Behav* 105, 193–198.

Safari, M.S., Haghparast, A., Semnanian, S. (2009). Effect of lidocaine administration at the nucleus locus coeruleus level on lateral hypothalamus-induced antinociception in the rat. *Pharmacol Biochem Behav* 92, 629–634.

Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P., Wilson, S., Arch, J.R., Buckingham, R.E., Haynes, A.C., Carr, S.A., Annan, R.S., Mc Nulty, D.E., Liu, W.S., Terrett, J.A., Elshourbagy, N.A., Bergsma, D.J., Yanagisawa, M. (1998). Orexins and orexin receptors: A family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92, 573–585.

Samineni, V.K., Premkumar, L.S., Faingold, C.L. (2011). Post-ictal analgesia in genetically epilepsy-prone rats is induced by audiogenic seizures and involves cannabinoid receptors in the periaqueductal gray. *Brain Res* 1389, 177–182.

Samson, W.K., Taylor, M.M., Ferguson, A.V. (2005). Non-sleep effects of hypocretin/orexin. *Sleep Med Rev* 9, 243–252.

Suplita, R.L., Farthing, J.N., Gutierrez, T., Hohmann, A.G. (2005). Inhibition of fatty-acid amide hydrolase enhances cannabinoid stress-induced analgesia: Sites of action in the dorsolateral periaqueductal gray and rostral ventromedial medulla. *Neuropharmacol* 49, 1201–1209.

Tasker, R.A.R., Choiniere, M., Libman, S., Melzack, R. (1987). Analgesia produced by injection of lidocaine into the lateral hypothalamus. *Pain* 31, 237–248.

Tsujino, N., Sakurai, T. (2013). Role of orexin in modulating arousal, feeding, and motivation. *Front Behav Neurosci* 7, 1–14.

Vaughan, C.W., McGregor, I.S., Christie, M.J. (1999). Cannabinoid receptor activation inhibits GABAergic neurotransmission in rostral ventromedial medulla neurons in vitro. *Br J Pharmacol* 127, 935–940.

Wallace, M.J., Blair, R.E., Falenski, K.W., Martin, B.R., DeLorenzo, R.J. (2003). The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy. *J Pharmacol Exp Ther* 307, 129–137.

Watanabe, S., Kuwaki, T., Yanagisawa, M., Fukuda, Y., Shimoyama, M. (2005). Persistent pain and stress activate pain-inhibitory orexin pathways. *NeuroReport* 16, 5–8.

Yamanaka, A., Muraki, Y., Tsujino, N., Goto, K., Sakurai, T. (2003). Regulation of orexin neurons by the monoaminergic and cholinergic systems. *Biochem Biophys Res Commun* 303, 120–129.